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During the second year of this grant, we obtained better X-ray data from the crystals of the T-cell receptor/HLA-A2/peptide complex, by finding improved conditions for freezing the crystals, by producing heavy atom-containing crystals, and by collecting data at the Cornell High Energy Synchrotron Source (CHESS). By the combination of the techniques of molecular replacement, multiple isomorphous replacement, and iterative real-space averaging, we were able to determine the structure of the TCR/HLA-A2/peptide complex. We are in the process of analyzing the immunological implications of the structure and are determining two closely related structures to allow a fuller interpretation of this central molecular recognition event in the immune system.

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David N. Barbagallo 21 Nov 96

PI - Signature Date

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Don C. Wiley DATE

Annual Report Table of Contents

ANNUAL REPORT (1 Sep 1995 - 31 Aug 1996)

Structural Analysis of the Human T-cell Receptor/ HLA-A2/Peptide Complex

Introduction

Cytotoxic T-lymphocytes play a vital role in the immune response to antigen by their ability to distinguish between self and foreign cells and subsequently to lyse foreign cells and/or to release cytokines. The basis for this differential recognition is the binding of the T-cell antigen receptor (TCR) to cell surface antigens in the form of peptide fragments bound to human leukocyte antigen (HLA) proteins of the major histocompatibility complex. Many tumors express tumor-specific antigens that can serve to label tumor cells as being "foreign" and that have been shown to direct specific lysis of tumor cells by cytotoxic T-lymphocytes (CTLs) in vitro (references 1-5). Little is known of the molecular interaction between a T-cell receptor and an HLA protein/peptide complex that leads to CTL responses. We are studying the interaction between a T cell receptor and a class I MHC/peptide complex by x-ray crystallography to gain an understanding of the molecular recognition involved in CTL responses.

In the second year of this grant, we determined the x-ray structure of a TCR/HLA-A2/Tax peptide complex. This has furthered our understanding of the recognition that is required for specific T-cell activation and may better inform the development of tumor immunotherapies using tumor-specific CTLs to control tumor growth.

Two papers describing the results obtained during this grant/fellowship have been published or are in press. They are listed in the references (references 6, 7) and preprints of the papers have been sent previously. I have been involved with an additional two papers (references 8, 9); preprints of those have also been sent.

In October 1995, Partho Ghosh, who is a postdoctoral fellow in the laboratory, joined the project. He is experienced in X-ray crystallographic techniques and analysis. Partho Ghosh and I (David Garboczi) collaborated in performing the work described in this report.

In the first year of this grant (described in the annual report of October 1995) we had obtained crystals. The human TCR and HLA-A2 proteins were produced in bacteria; procedures for producing abundant HLA-A2 complexed with single peptides had been available from my previous work (10). Refolding experiments with the TCR obtained from bacterial expression had been successful and ample TCR protein was in hand (7). The TCR protein (75 mg/ml) and HLA-A2/Tax protein (12 mg/ml) were mixed at a 1:1 molar ratio and set up in a hanging drop vapor diffusion crystallization experiment using a screen of crystallization conditions. Crystals appeared in 10% polyethylene glycol (PEG), 100 mM Mg acetate, 50 mM NaCacodylate, pH 6.5. These crystals were reseeded (11) and large (200 x 200 x 100 μm) crystals were obtained. The larger crystals were mounted in a glass capillary tube and diffraction was observed to about 3.5 Å. Crystals were placed into the above buffer plus 20% glycerol and frozen at -160°C. Diffraction was observed to about 3.3 Å and a complete dataset was measured to 3.5 Å (60% complete to 3.3-3.4 Å). The spacegroup of the crystals is C2, with unit cell dimensions, a=228.2 Å, b=49.6 Å, c=94.7 Å, and β=91.3°.

Results

We had some difficulty in freezing diffraction-quality crystals reproducibly, but found improved conditions for freezing the crystals using 20% glycerol and 50 mM TrisCl, pH 8.5 with 10% polyethylene glycol and 100 mM Mg acetate. Crystals frozen under these conditions reproducibly diffracted X-rays. Microseeded crystal growth was performed at a PEG concentration of 7.5%, which appeared to

increase the size and number of crystals obtained. The size of a typical crystal used for data collection was 100 x 200 x 700 μm.

We confirmed the C2 spacegroup based on the dataset described in the annual report of last year. Analysis by XDS (12) autoindexing and inspection of integrated, but unreduced intensities in HKLVIEW (13) showed the spacegroup to be C2. The R_{sym}= 12.4 % and the mosaicity was estimated to be 0.6 deg full-width at half-height from analysis of a rocking curve. From the cell constants, one complex was estimated to be in the asymmetric unit with a 56% solvent content.

Using the program AMORE (13), a molecular replacement (MR) solution with the HLA-A2/Tax model (14) was readily found with a correlation coefficient (cc) of 0.29 in the rotation search (next highest cc = 0.16) and with a cc in the translation search of 0.29, and an R-factor (Rf) of 51% (next highest cc = 0.21, Rf = 54%). Translation solutions were found with a mouse β subunit model (15) with a cc of 0.37 and a Rf of 0.49) and with a mouse Vα domain model (16) with a cc of 0.44 and a Rf of 46.6% (next highest cc= 0.42, Rf- 47.4%). Rotation searches were done with 10-3.8 Å data and translation searches were done with data from 15-3.8 Å. The β and α search models were poly-alanine except where residues were conserved. The mouse β subunit has 69 % sequence identity and Vα has 34 % sequence identity with this human TCR. Some loops in Cβ and in Vα were deleted for the search. Molecular replacement translation functions with α and β domains were done with HLA-A2 fixed. The MR placement of the V regions "looked right" as they independently were positioned above the binding site and formed an eight-stranded beta barrel at their interface, being very similar to the structure of the V-V portion of antibody fragments (Fab).

A 2Fo-Fc electron density map using just HLA-A2 as model to generate Fcalcs and solvent-flattened and histogram-matched with the program DM (13) revealed density above the peptide binding site, but this and other 2Fo-Fc maps

using various combinations of the A2 and TCR domains as models were poorly interpretable for portions of the protein that were not part of the models.

Synchrotron data collection I

We planned to obtain a better X-ray dataset and believed that we'd likely solve the structure with higher resolution data and the HLA-A2, mouse TCRα, and TCRβ models. We also planned to collect data from crystals soaked in mercury and platinum to make heavy atom derivatives for use in multiple isomorphous replacement (MIR). A mutation was made in the Tax peptide (sequence: LLFGYPVYV) by changing the leucine at position 1 to a cysteine (P1cys). The TCR/HLA-A2 gel shift still occurred with the P1cys peptide. The HLA-A2/P1cys protein was ready at the last minute before going to the synchrotron and had not been purified. At the Cornell High-Energy Synchrotron Source (CHESS) we were able to purify the protein by precipitating impurities with 20 mM Mg acetate and at the pH 6.5 of the crystallization solutions. Micro-seeded drops with TCR and the precipitation-purified HLA-A2 yielded crystals there, at CHESS. They were treated with ethyl mercury phosphate and the mercury derivative data was immediately collected. Native crystals were treated with K_2PtCl_4 for the platinum derivative.

Native data (data resolution limit 2.8 Å) and derivative data (mercury 3.4 Å and platinum 3.6 Å) were obtained at CHESS on an Area Detector Systems Corp (ADSC) charge-coupled device (CCD) X-ray detector, and were integrated and scaled with DENZO/SCALEPACK (Z. Otwinowski, personal communication). The P1cys mercury data was the least mosaic (0.6 %) but not complete (75 %). Native data was 79% complete and the Pt data was 94% complete, but both were highly mosaic (1.5°and 1.4°, respectively). The mercury position was found by difference Patterson methods. Its location was confirmed in a difference Fourier map phased on HLA-A2; the mercury peak was located on the side chain of P1 of the peptide. The platinum positions were located in difference Fourier maps. Refinement of

heavy atom positions in MLPHARE (13) yielded one major and one minor site (phasing power = 0.94, >1 to 4.9 Å, R_{cullis}= 0.76 to 3.5 Å) for the mercury and one major and four minor sites (phasing power= 0.92, >1 to 4.9 Å, R_{cullis}= 0.82 to 3.5 Å) for the platinum, with an overall figure-of-merit of 0.35. The positions and occupancies of the heavy atom positions were re-calculated in MLPHARE using DM-modified phases as reference phases. Solvent-flattened and histogram-matched MIR electron density maps showed broken and ambiguous density above the peptide-binding site. The synchrotron native dataset did not scale well with the first dataset that was collected in the laboratory on a multiwire detector (Siemens) detector (R_{deriv} = 40 %).

Synchrotron data collection II

We decided to obtain datasets on additional derivatives to improve the MIR results and also to attempt to obtain a better native dataset. Single and double cysteine mutants of β_2-microglobulin (β_2m) were made by Qing Fan, a graduate student in the laboratory, at positions 52, 67, 91 in β_2m chosen to be accessible to solvent and to be free of crystal contacts by study of the packing model from the MR placement of the models. The refolding of HLA-A2 was not impaired by the cysteine mutations in β_2m. To provide an additional mercury site to generate an additional derivative, an eleven amino acid peptide with cysteine at P1 and P11 and glycine at P10 was made; the TCR/HLA-A2 gel shift still occurred.

Data was obtained at CHESS with the same CCD detector. Derivative datasets obtained during the second trip to CHESS were named by the position of free cysteines placed for mercury binding: P1cys, Y67C K91C, P1P11, native, K91C. Some of the datasets would not scale well with the other datasets and we found that the data fell into two groups. Within a group the data would scale between datasets and achieve a R_{deriv} of 20-25 %. Between the two kinds of crystals, the data would not scale well (R_{deriv} of 40 %, 35-40% even in the low resolution bins), even

though the cell constants are almost the same. The cell constants for the two best native sets are (a=228.4 Å, b=49.4 Å, c=95.7 Å, β= 89.7°; α=229.3 Å, β=49.5 Å, c=96.0 Å, β= 89.6°). For group 2, the Y67C K91C set (called B5) was chosen as "native" (2 mercury atoms) and is phased by the sets: native, K91C, and P1cys. The B5 data is to 2.6 Å with a 1.2° mosaicity and a completeness of 90%. We found that there were rotation and translation differences in domain positions between the two native sets approximately as given:

Domain	Rotation	Translation
α1α2	2.0°	2.9 Å
α3	3.2	4.8
β_2m	0.8	1.5
Vβ	3.2	4.6
Cβ	2.4	3.8
Vα	1.8	2.6

Averaging the two MIR maps generated from the two groups of data was begun. Initial transformations between the seven domains were obtained from the MR solutions. To improve the transformations, domain by domain density correlation between the two maps was performed with the IMP program from the RAVE package (17). The 2Fo-Fc map using HLA-A2 as model was used in the averaging as a real space contribution to the averaging and in parallel, in phase combination with the MIR phases of each cell to contribute to the starting maps. The HLA-A2 molecular replacement map was allowed to contribute to just its own masks or to all the masks. Averaging was performed in two ways, either phase-combining the averaged phases with the MIR phases at each cycle or not. Current rotation/translation operators were obtained by refining the model into each dataset and determining the tranformation in O (ref. 18).

Two-fold averaged MIR/MR maps were much improved and allowed the positioning and building of all the main chain except the CDR3 regions and most of the side chains of the Vα and Vβ domains. 2Fo-Fc maps using this improved model allowed a convincing trace of the CDR3 regions.

The new space group $P2_1$ with the 11mer peptide had the same cell constants but no centering and therefore had two complexes per asymmetric unit. Deciding between the P2 and $P2_1$ spacegroups was difficult ; there were very few observations of 0k0 reflections since k is along the 49 Å axis. Molecular replacement with AMORE placed HLA-A2 in one of the two positions, but did not find the second position. A difference Fourier using HLA-A2 as model (in only one of the two positions in the asymmetric unit) located the two mercury atoms in the asymmetric unit. The second HLA-A2 model was translated to match the second mercury site. Top rotation/translation solution assuming $P2_1$: cc= 0.29, Rf= 51% and the packing looked possible,and top rotation/translation solution assuming P2: cc= 0.25, Rf=52% and the molecular packing was impossible as the models overlapped each other. The $P2_1$ information was included in the real-space averaging making the averaging four-fold.

Description of the Structure

The 2.6Å structure (see ref. 6 for more details and figures) of the TCR/Tax/HLA-A2 complex shows that the TCR is oriented diagonally over the HLA-A2/peptide complex, with CDR1 and CDR3 from both Vα and Vβ contacting the peptide. This orientation appears similar to that in the preliminary description of a mouse TCR (2C)/peptide/H-$2K^b$ complex (19). The overall structure of the TCR in the TCR/Tax/HLA-A2 complex is similar to the structure of a Fab. All three variable loops of Vα and CDR3 of Vβ contact conserved and polymorphic positions on the α-helices of the MHC molecule. The interface between TCR and

MHC/peptide is in unambiguous electron density, as are the α1, α2, and β_2m domains of the MHC and the Vα and Vβ domains of the TCR.

Figure 1 (see Appendix) shows the TCR/Tax/HLA-A2 complex and how the TCR (top) and MHC molecule (bottom) would span the space between a CTL and virally infected target cell.

When the peptide-binding site of HLA-A2 is viewed from above, the TCR is oriented diagonally across the site (Figure 2 in Appendix). The TCR third variable loops of both Vα and Vβ meet at the center of the binding site over the center peptide residue Y5. The CDR1 loops are also positioned over the peptide. CDR1α extends from the peptide N terminus to Y5, and CDR1β is over the C-terminal end of the peptide near Y8. The second variable loops are over the MHC molecule, CDR2α over the α2 domain α-helix and CDR2β over the α1 domain α-helix.

Almost the entire Tax peptide is buried in the TCR/MHC interface. The TCR makes contacts with peptide residues L1, L2 and from G4 to Y8. Y5 is bound in a deep pocket at the center of the TCR where the CDR3 loops converge, reminiscent of the structures of peptides complexed with anti-peptide Fabs (reviewed in ref. 20). The peptide is bound much more deeply in the MHC molecule than it is in the TCR.

The diagonal orientation of the TCR allows the flat surface of the TCR to interact with the peptide by fitting down between the two highest points on the MHC molecule, toward the N terminal of the α1 domain α-helix and toward the N-terminal end of the α2 domain α-helix, a feature of the diagonal orientation also recognized by Nathenson and colleagues (21). The high points of the α2 domain α-helix and the α1 domain α-helix are a topographical feature common to all class I and class II molecules.

No gross conformational change of the TCR was observed in the complex relative to the structures of isolated TCR domains or Fabs that might indicate that it could send information to the cytoplasm that it had bound antigen.

Conclusions

The suggestion that a diagonal orientation allows a general 'interlocking' binding mode between TCR and MHC was first proposed on the basis of mutations on mouse class I molecules (21). In addition to the structural arguments presented here, functional arguments have been made in favor of a general binding mode (refs 22, 23 for example). For example, it would provide a molecular mechanism for an inherent bias of TCR for self-MHC (24) and, coupled with the apparent physical limit on TCR specificity for peptide seen in this structure, helps to explain the ability of one peptide positively to select a nearly normal TCR repertoire (23,25). A general TCR binding mode would simplify the processes of selection during repertoire development and provide an explanation for the magnitude of the alloreactive response. One way that the danger inherent in hypervariable molecules may be controlled is by restricting their opportunity for cross-reactivity to a single binding mode on MHC molecules, which could be part of the basis of mechanisms that maintain immunological tolerance.

Plans for the Third Year of the Fellowship

As we continue to analyze the TCR/Tax/HLA-A2 structure for additional insights, we are also determining the structures of two related complexes to more fully understand the recognition of the antigenic peptide/MHC complex. We have identified two mutant Tax peptides that alter the TCR recognition of the HLA-A2 complex (see ref. 7 for complete details). One of them, Y5A, allows near wild-type cell-killing by the T cell bearing the anti-Tax receptor, but promotes less than 10% of

the wild-type cytokine release by the T cells. Y5A promotes complex formation as seen in a gel shift assay; a shifted band is observed. The partially-activating characteristic of this peptide has led peptides of this sort to be termed "partial agonist" peptides (26). We have TCR/Y5A/HLA-A2 crystals that diffract x-rays. The other peptide contains the mutation Y8A and, in T cell assays, is inactive, promoting neither cell-killing nor cytokine release. There is not a gel shift with Y8A. Yet crystals that diffract x-rays form from a mixture of HLA-A2/Y8A and TCR.

Preliminary x-ray data from these crystals show them to be isomorphous with the crystals containing wild-type peptide. If they are not isomorphous enough to simply use the phases from the solved structure, we should be able to readily solve, in the third year of this grant, the two new structures by molecular replacement techniques.

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Table I. Structural analysis

Data Sets	Resolution limit (Å)	Mosaicity	R_{merge}‡	Comp† (red)	I/σ_I	R_{deriv}‡ (Sites)	R_{cullis}‡	f_h/ε§	fom†† (res)
A. C2 (Z=1)*									0.41 (3.5 Å)
Native	2.90	2.5°	13.9	82 (3.7)	7.3				
L1C + EMP	3.10	1.0°	10.1	98 (2.5)	8.2	22.4 (1)	75	1.13	
K91C + EMP	3.20	2.4°	8.6	81 (2.7)	9.2	21.6 (1)	89	0.79	
Y67C/K91C + EMP	2.60	1.1°	10.9	95 (3.1)	7.0	27.2 (2)	85	0.94	
B. C2 (Z=1)*									0.35 (3.5 Å)
Native	2.80	1.5°	11.8	79 (1.8)	6.4				
L1C + EMP	3.40	0.6°	10.7	75 (1.8)	6.3	23.5 (2)	76	0.94	
K_2PtCl_4	3.60	1.4°	11.8	93 (2.5)	7.0	34.5 (5)	82	0.92	
C. $P2_1$ (Z=2)*									0.30 (3.7 Å)
L1C/11C Native	3.75	3.0°	14.3	86 (2.6)	6.6				
L1C/11C + EMP	3.40	2.5°	13.9	85 (2.6)	6.5	35.4 (2)	80	1.12	

Molecular Replacement§§	HLA-A2/Tax9	TCR Vα	TCR Vβ	TCR Cβ
Rotation (10-3.8 Å)	7.3σ	2.3σ	4.3 σ	3.4σ
Translation (15-3.8 Å)	10.3σ	8.5σ	10.3σ	5.7σ

Refinement Resolution	Reflections (free)	Atoms	R_{free}‡	R_{cryst}‡	Bonds‡‡	Angles‡‡	B-factors¶
6.0-2.6 Å	26273 (3006)	5716	32.2	24.0	.013 Å	1.81°	4.8 Å^2

Domains	$\alpha_1\alpha_2$	α_3	β_2m	Tax9	Vα	Vβ	Cβ
Mean B-factors	27 ± 15 Å^2	81 ± 32 Å^2	29 ± 16 Å^2	19 ± 7 Å^2	36 ± 15 Å^2	36 ± 15 Å^2	65 ± 18 Å^2
Real-Space Fit**	0.87 ± .03	0.74 ± .15	0.86 ± .09	0.89 ± .02	0.87 ± .05	0.88 ± .03	0.80 ± .12

*Space groups of crystal forms and derivatives are described in Methods. Z, number of MHC/Tax/TCR complexes per asymmetric unit.

‡$R_{merge} = 100 \times \frac{\sum_h \sum_i |I_{h,i} - I_h|}{\sum_h \sum_i I_{h,i}}$, where I_h is the mean intensity of symmetry-related reflections, $I_{h,i}$.

$R_{der} = 100 \times \frac{\sum_{hkl} | F_p - F_{ph} |}{\sum_{hkl} F_p}$, where F_p is the native amplitude and F_{ph} is the derivative amplitude. Sites, number of heavy atom sites per asymmetric unit.

$R_{Cullis} = 100 \times \frac{\sum_{hkl} | | F_p \pm F_{ph} | - F_{h\,calc} |}{\sum_{hkl} | F_p \pm F_{ph} |}$, where F_p is the native amplitude, F_{ph} is the derivative amplitude for centric reflections, and $F_{h\,calc}$ is the calculated heavy atom structure factor.

$R_{free} = 100 \times \frac{\sum_{hkl} | F_{obs} - F_{calc} |}{\sum_{hkl} F_{obs}}$, where R_{free} is calculated for a randomly chosen 10% of reflections ($F > 0$) omitted from refinement, and R_{cryst} is calculated for the remaining 90% of reflections ($F > 0$) included in refinement.

†Comp = 100 x (number of observed unique reflections)/(number of possible unique reflections). red = (number of observed reflections)/(number of observed unique reflections).

§ fH/e is the phasing power, with fH being the mean amplitude of the heavy atom structure factor and e the r.m.s lack-of-closure error.

††fom, figure of merit. res, resolution to which figure of merit is determined.

§§Rotation and translation solution peak heights above the mean in rotation and translation function maps.

‡‡r.m.s. deviations from ideal values.

¶r.m.s. deviation between B-factors of bonded atoms.

**Real-space fit correlation coefficient [45] calculated from 2Fobs-Fcalc electron density and the refined model of MHC/Tax/TCR.

TABLE 1 (continued)

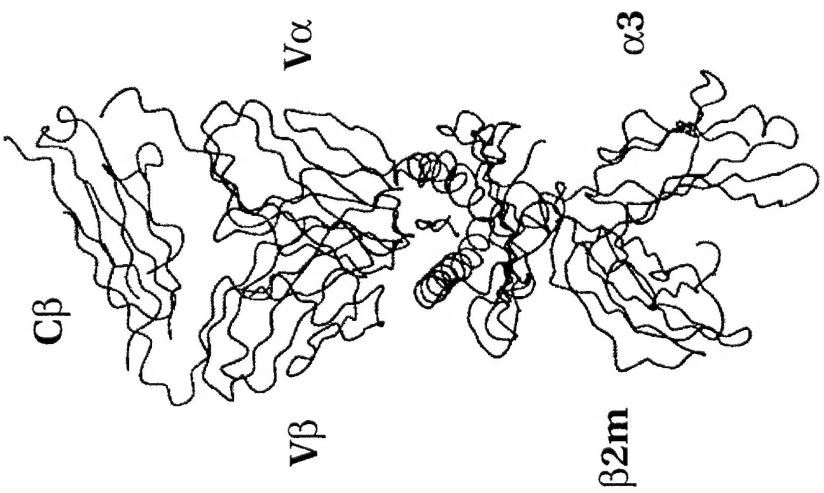

Fig. 1. Intercellular complex of alpha/beta TCR (top) and the HTLV-1 Tax peptide bound to HLA-A2 (bottom) shown as an alpha-carbon trace, colored by temperature factor: blue is low and red is high temperature factor (greater than 60 square angstroms) and shows less ordered regions. The TCR constant region of alpha is not shown.

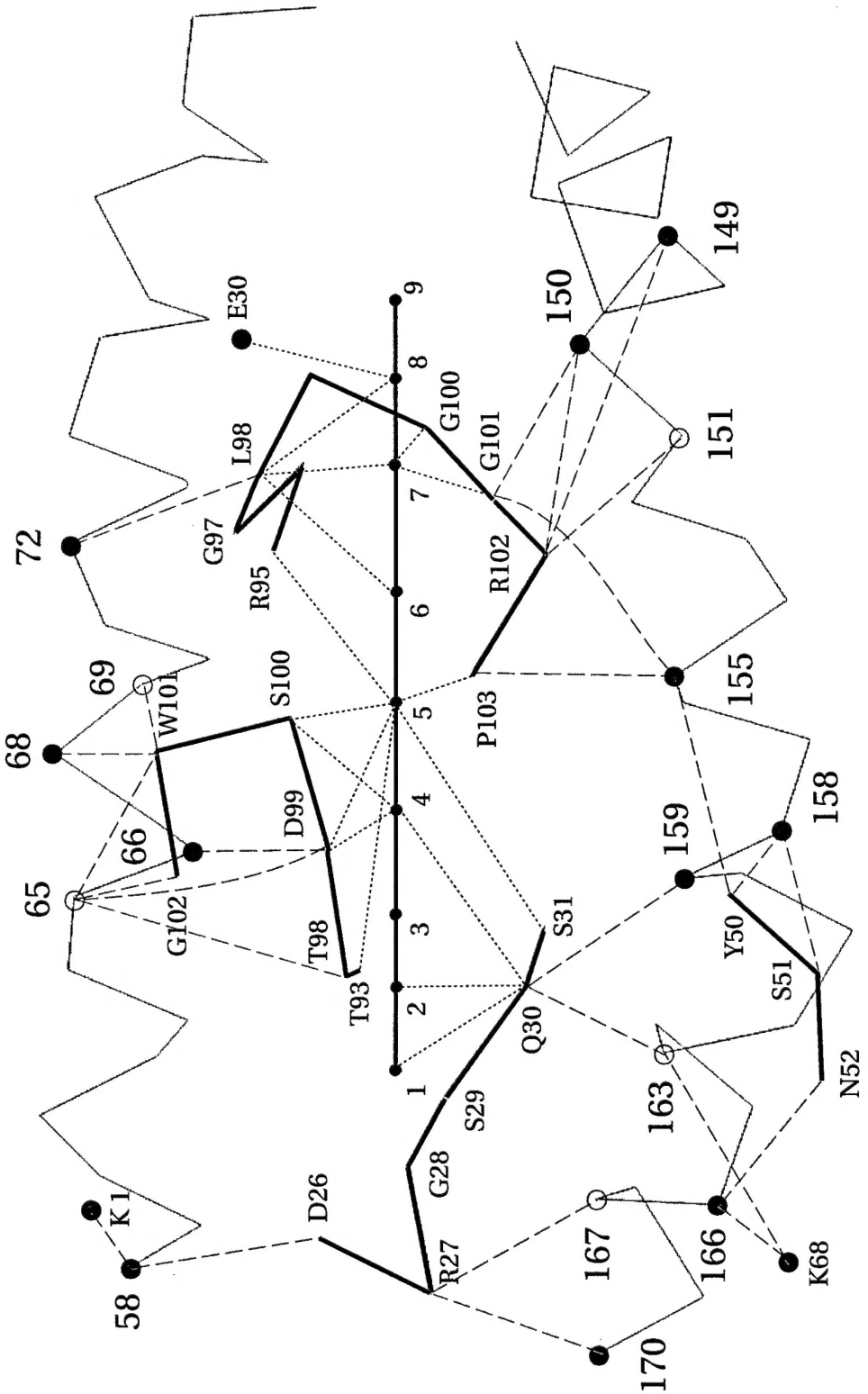

Fig. 2. Interaction of TCR with the Tax peptide and HLA-A2. CDR residues (solid red lines) contact HLA-A2 (green dashed lines) and Tax peptide (red dotted lines). Human MHC class I conserved (solid circles) and polymorphic (open circles) residues are shown. For clarity, the peptide is depicted as a straight line and contact between Valpha30Q and HLA-A2 is not shown.